RESEARCH ARTICLE

In-Vitro Antioxidant, Antibacterial and Anticancer properties of Leaf Extract *Annona Muricata*

P.Gowsalya*, T.Sathya
Department of Biochemistry, Navarasam Arts and Science College for Women, Arachalur, Erode-638101, Tamil Nadu, India

ABSTRACT: *Annona muricata* is one of the important herbal plant that are widely used to treat antidiabetic, anti-inflammatory, insecticidal, antimalarial, anticancer, antibacterial and antioxidant activities. In the present study, extraction procedure was carried out in few step processes method. The extract contains high percentage of steroid, alkaloid, flavonoid, phenolic and saponin. The extract was used to study the antioxidant activity; antibacterial activities and anticancer activity by standard methods. The antioxidant activity was studied by using radical scavenging DPPH, FRAP and H2O2 method and disc diffusion method. The results of antibacterial activity of the following bacteria's such as Pseudomonas, Staphylococcus, Bacillus, E.coli shows the maximum zone of inhibition against Staphylococcus aureus which is around 1.7 cm. The anticancer activity was carried out by MTT assay using Hep-G2 as cell line and results are reported in the paper.

KEYWORDS: Annona muricata , phytochemicals, antioxidants, micro organisams, anticancer

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INTRODUCTION

Medicinal plants are considered as the basis for health preservation and care worldwide. Chronic degenerative diseases (diabetes, cardio-vascular and cancer) have reached epidemic proportions and are considered as a serious health problem; therefore, the treatments of these diseases are of clinical importance [1]. *Annona muricata* is a lowland tropical evergreen fruit tree, belonging to the Annonaceae family comprising approximately 130 genera and 2300 species, and is widely grown and distributed in tropical and subtropical regions around the world. The aerial parts of graviola have several functions: the fruits have been widely used as food confectionaries, while several preparations, especially decoctions of the bark, fruits, leaves, pericarp, seeds, and roots, have been extensively used in traditional medicine to treat multiple ailments including cancers by local communities in tropical Africa and South America [2]. This plant is commonly known as Soursop, Prickly custard apple, Brazilian pawpaw, Guanabana,, Graviola (English), Mullu-sitha-pazham (Tamil) [3].

The soursop tree is about 5–10 m tall and 15–83 cm in diameter with low branch [4],[5]. It tends to bloom and fruit most of the year, but there are more defined seasons depending on the altitude. It is distributed in the tropical regions of Central and South America, Western Africa and Southeast Asia , at altitudes below 1200 m above sea level, with temperatures between 25and 28°C, relative humidity between 60 and 80%, and annual rainfall above 1500 mm. The soursop fruit is an edible collective ovoid berry, dark green in colour. Its average weight is4 kg in some countries [6], but in Mexico [4], Venezuela [7] and Nicaragua [3], it ranges between0.4 and 1.0 kg. Each fruit may contain 55–170 black seeds [8] when fresh and they turn light brown when dry. The flesh is white and creamy with a characteristic aroma and flavor [6].

A. muricata has been used in mainly developing countries for the treatment of arthritis[9], hypertension[10] , snake bite[11] , diarrhea [12], headache[13] , and malaria[14] . In addition, it has been mentioned as an antimicrobial [15], anti diabetic [16], anti-inflammatory [17], anti protozoan [18] , antioxidant [19], insecticide [20] , larvicide , and anticancer[21]. In spite of the importance, the present study has been undertaken to extract the plant compounds and studied their anti-bacterial and anti-cancerous activity by in-vitro methods.

MATERIALS AND METHODS

Materials

Collection of sample

**Corresponding Author: gowsiguruvishnu@gmail.com*
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The leaves of *Annona muricata* are collected from the outskirts of Pongalur (Tiruppur,Tamil Nadu,India)

METHODS

Preparation of plant extract

The leaves are washed in water to remove the dust and other impurities. Then the leaves were shade dry for about 10-15 days. The dried leaves were coarsely powdered using an electric mixer and then subjected to extraction using soxhlet apparatus. The extract was then stored and used for further study.

Phytochemical Screening

Phytochemical screening for alkaloids, flavonoids, reducing sugar, phytosterols, steroids, saponins,tannins and cardiac glycoidies were performed according to the standard procedures described by [22] .

In-vitro anti-oxidant activity

DPPH Radical Scavenging Test

The free radical scavenging activity of ethanolic extract of *Annona muricata* was measured by using 2,2-diphenyl-1-picrylhydrazyl (DPPH). The scavenging activity for DPPH free radical was carried out according to the procedure described by Braca[23]. An aliquot of 3ml 0.004% DPPH solution in methanol and 0.5 to 2.5 µl of plant extract at various concentration various concentration were mixed. The mixture was shaken vigorously and allowed to reach a steady state at room temperature for 30 min. Decolourization of DPPH was determined by measuring the absorbance at 517 nm. A control was prepared using 0.1 ml respective vehicle in the place of plant extract. The percentage inhibition of DPPH radicals by the extract was determined by comparing the absorbance values of the control and the experimental tubes.

% inhibition =[Abs(control)- Abs(sample)/ Abs(control)] x 100

Ferric reducing antioxidant power (FRAP) assay

The antioxidant property of the plant extract samples were estimated according to the procedure described by [24]. FRAP reagent (900µl), prepared freshly and incubated at 37^{o}C, was mixed with 90µl of distilled water and 30µl of test sample, or methanol (the reagent blank). The sample and reagent blank were incubated 37^{0}C for 30 minutes in water bath. The FRAP reagent contained 2.5 ml of 20mmol/l TPTZ solution in 40mmol/l HCl plus 2.5 ml of 20mmol/ $FeCl_3.6H_2O$ and 25 ml of 0.3 mol/l acetate buffer , pH 3.6 [24]. At the end of incubation period the absorbance reading was recorded immediately at 593nm using a spectrophotometer. The known Fe(II) concentration ranging between 100 and 2000 µmol/l ($FeSO_4.7H_2O$) was used for the preparation of calibration curve. The parameter Equivalent Concentration (EC1) was defined as the concentration of antioxidant has ferric- TPTZ reducing ability equivalent to that 1 mmol/l $FeSO_4.7H_2O$. EC1 was calculated as the concentration of antioxidant giving an absorbance increase in the FRAP assay equivalent to the theoretical absorbance value of 1 mmol/l concentration of Fe(II) solution determined using the corresponding regression equation.

% inhibition =[Abs(control)- Abs(test)/Abs(control)]X 100

Hydrogen peroxide scavenging assay

The free radical scavenging activity of the leaf extracts were determined by using Hydrogen peroxide assay [25]. In a clean dry test tube about 1ml of hydrogen peroxide was taken as a control. Then the experimental sample tubes were added in five different concentrations such as 50µl. 100µl, 150µl, 200µl, 250µl. Then the H2O2 reagents were added in all the test tubes. Allow all the test tubes for incubation for about10 minutes in dark at room temperature. Then the absorbance of samples was read at 610nm

% Inhibition = [(Abs(control)- Abs(sample) / Abs(control)] × 100

In-vitro anti-bacterial activity

Preparation of the bacterial inoculums

Active culture for the experiments were prepared by sample containing test culture to test tube of 50 ml nutrient broth and the bacterial culture were incubated with agitation for 24 hours and at 37^{0}C in shaking incubator.

Agar well diffusion method

The antibacterial activity of them extracts was determined by Well Diffusion method MHA plates were prepared by pouring 20 ml 0f molten media into sterile plates. After solidification of media, 20-25 µl test culture was swabbed uniformly on Mullur Hinton Agar plates. On each inoculated plate, 10 mm diameter wells (5 wells at equal distance in one plate) were bored on the agar using sterile cork borer. Concentration at 25µl, 50µl, 75µl and 100µl of extracts was added to each

well by sterile Pasteur pipette and allowed to diffuse for 1 hr, before incubating the plates for 18 to 24 hours at 37°C. Then plates were incubated at 37° C about 24 hours and control was also maintained. The diameter of the inhibition zone resulting from the activity of the plant extract was measured in cm, activity was recorded. The antibacterial activity of the plant extract against the selective microorganism was evaluated and compared with that of the antibiotic chloramphenicol [26].

IN- VITRO ANTICANCER ACTIVITY

Cell line

The human Liver cancer cell lines (Hep-G2) was obtained from National Centre for Cell Science (NCCS), Pune and grown in Eagles Minimum Essential Medium containing 10% fetal bovine serum (FBS). The cells were maintained at 37°C, 5% CO2, 95% air and 100% relative humidity.

Cell treatment procedure

The monolayer cells were detached with trypsin-ethylene diamine tetra acetic acid (EDTA) to make single cell suspensions and viable cells were counted using a hemocytometer and diluted with medium containing 5% FBS to give final density of $1x10^5$ cells/ml. One hundred micro litres per well of cell suspension were seeded into 96-well plates at plating density of 10,000 cells/well and incubated to allow for cell attachment at 37°C, 5% CO_2, 95% air and 100% relative humidity. After 24 h the cells were treated with serial concentrations of the test samples. They were initially dissolved in neat dimethylsulfoxide (DMSO) and an aliquot of the sample solution was diluted to twice the desired final maximum test concentration with serum free medium. Additional four serial dilutions were made to provide a total of five sample concentrations. Aliquots of 100 µl of these different sample dilutions were added to the appropriate wells already containing 100 µl of medium, resulting in the required final sample concentrations. Following sample addition, the plates were incubated for an additional 48 h at 37°C, 5% CO_2, 95% air and 100% relative humidity. The medium containing without samples were served as control and triplicate was maintained for all concentrations. [27]

MTT assay

The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl- -tetrazolium bromide (MTT) is a yellow water soluble tetrazolium salt. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore,the amount of formazan produced is directly proportional to the number of viable cells.

After 48 h of incubation, 15µl of MTT (5mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37°C for 4h. The medium with MTT was then flicked off and the formed frozen crystals were solubilized in 100µl of DMSO and then measured the absorbance at 570 nm using micro plate reader. [28]

The percentage cell viability was then calculated with respect to control as follows:

% Cell viability = [A] Test / [A]control x 100

The % cell inhibition was determined using the following formula:

% Cell Inhibition = 100- Abs (sample)/Abs (control) x100.

Nonlinear regression graph was plotted between % Cell inhibition and Log concentration and IC50 was determined using Graph Pad Prism software.

RESULTS AND DISCUSSION

Phytochemical Analysis

The results of phytochemical analysis of *Annona muricata* was reported in Table.1. The Phytoconstituents such as Phenols, Flavonoids, Tannis, Cardiac glycosides were highly present in the samples. The Alkaloids, Reducing sugar, Phytosterols, Steroids were also moderately present and Sopanins was also present in small quantities. Alkaloids are naturally occurring chemical compounds containing basic nitrogen atoms. They are often having pharmacological properties and are used as medications and recreational drugs [29]. Flavonoids enhance the effects of Vitamin C and function as antioxidants. They are also known to be biologically active against liver toxins, tumors, viruses and other microbes. Plant terpenoids are used extensively for their aromatic qualities. They play an important role in traditional herbal studies and are under investigation for Antibacterial, Anti neoplastic and other Pharmaceutical functions [30]. Tannins have shown potential Antiviral, Antibacterial and Anti parasitic effects. Saponins trigger hemolysis of red blood cells [31].Cardiac glycosides are drugs used in the treatment of congestive heart failure and cardiac arrhythmia. Most phytochemicals have antioxidant activity and protect our cells against oxidative damage and reduce the risk of developing certain types of cancer. The present study on *Annona muricata* results were in accordance with the work done by [32].

Table 1: Phytochemical analysis of the ethanolic extract of *Annona muricata*

S.No	Phytoconstituents	*Annona muricata*
1	Alkaloids	++
2	Phenols	+++
3	Reducing sugar	++
4	Saponins	+
5	Flavonoids	+++
6	Phytosterols	++
7	Amino acid and Protein	-
8	Steroids	++
9	Tannins	+++
10	Cardiac glycosides	+++

+' present, '**+++**' moderately present ,
'**+++** ' highly present '**-**' Absence

IN VITRO ANTI OXIDANT ACTIVITY

Determination of Radical Scavenging Activity by DPPH Method

The results of anti-oxidant activity of the plant extract using DPPH method using ethanol as solvent is shown in Table 4 and Figure 1.

Table 4: The anti-oxidant activity of *Annona muricata* using ethanolic extract

Concentration	Standard (Ascorbic acid)	DPPH Sample
0.5 µl	15 %	16%
1.0 µl	38 %	30%
1.5 µl	56 %	74%
2.0 µl	73 %	80%
2. 5 µl	86 %	92%

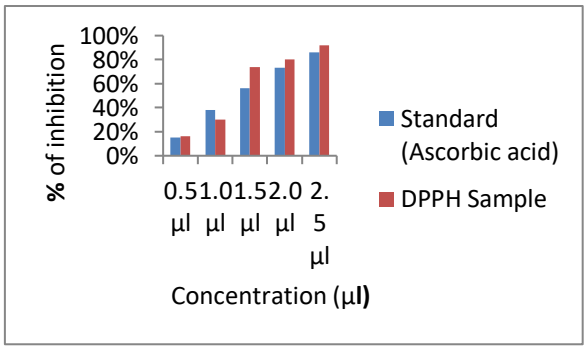

Figure: 1 Anti-oxidant activity of *Annona muricata* by DPPH

Determination of Radical Scavenging Activity by FRAP

Table 5: The anti- oxidant activity of *Annona muricata* using ethanolic extract

concentration	FRAP Standard	FRAP Sample
0.5 µl	33 %	53%
1.0 µl	44 %	56%
1.5 µl	52 %	63%
2.0 µl	71 %	73%
2. 5 µl	83 %	82%

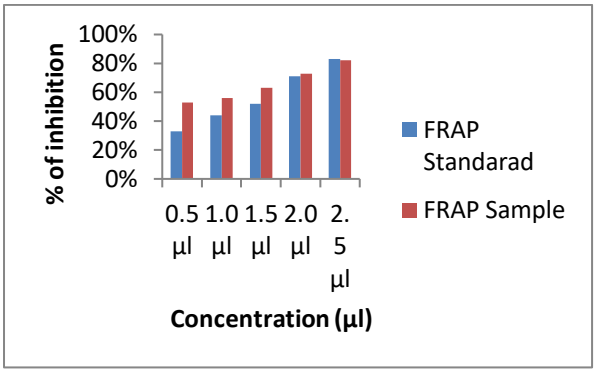

Figure: 2 Anti-oxidant activity of *Annona muricata* by FRAP

Determination of radical scavenging activity by H_2O_2 assay

Table 6: The anti- oxidant activity of *Annona muricata* using ethanolic extract

Concentration	Standard (Ascorbic acid)	Sample
0.5 µl	32 %	36%
1.0 µl	48 %	42%
1.5 µl	55 %	66%
2.0 µl	72 %	70%
2. 5 µl	87 %	81%

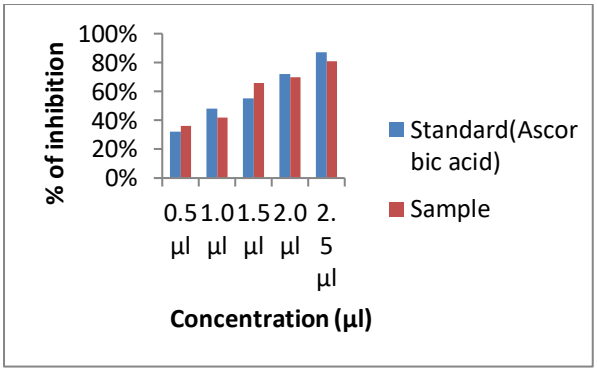

Figure:3 Anti-oxidant activity of *Annona muricata*by H_2O2

The results show that the antioxidant activity in DDPH, FRAP and Hydrogen Peroxide scavenging assay is found to more in the concentration of 2.5µl. Phenolic substances are pharmacologically active components of plants which are capable of neutralizing free radicals, chelating metal catalysts and inhibiting the activity of oxidizing enzymes in biological systems [33]. The antioxidant activity DPPH and FRAP of the soursop leaves inoculated with the pathogens allowed to see a marked difference concerning the control fruits. Liu et al. [34] showed that the high antioxidant capacity of soursop leaves is due to the high polyphenols content, which blocks oxidation and activates other antioxidants. The increase of antioxidant activity may be related to the secondary responses of soursop leaves to defend against an infective process, which includes an elevation of the levels of constitutive defensive toxins, of pathogen receptors and structural reinforcement of cell walls in tissues. The process determines a substantial change in the metabolic profile of the active cells [35].

In-vitro anti-bacterial activity

Antibacterial activity was performed by agar diffusion method. The stock culture of bacteria (*Pseudomonas, Staphylococcus*, *Bacillus, E.coli*) were received by inoculating in nutrient broth media and grown at 37% for 18 hours. The agar plates of the above media were prepared. Each plate was inoculated with 18 hours old cultures the bacteria were swab in the sterile plates. Cut the 5 wells Pour the plant extract in ratio 25µl, 50µl, 75µl, 100µl. All the plates were incubated at 37^{o}C for 24 hours and the diameter of inhibition zone was noted in cm. Agar well diffusion method has been used to determine the antimicrobial activities and minimum inhibitory concentrations or plant extracts against Gram positive, Gram negative bacteria. The extracts exhibited antibacterial activities against tested microorganisms [36].

The medicinal plant *Annona muricata leaf extract* showed good antibacterial activity against several organisms like *Staphylococcus a*, *Pseudomonas, Bacillus*, and *E.coli* as supported by previous studies [37], Antibacterial screening showed that the maximum zone of inhibition was noted for ethanolic extract against *Staphylococcus aureus* (1.7 cm).The present study on Annona muricata results were in accordance with the work done by Torres [37]

In-vitro Anti-Cancer Activity MTT Assay

The result of MTT assays revealed that the plant extract was decreased the percent viability of all the cells but to different extent. Plant extract was found to induce more cytotoxicity towards cancer cell lines. These results revealed morphological changes and shrinkage of cells leading to cell death induced by the extracts in the Liver cancer cell lines. The IC_{50} values of plant extracts of against the cancer cell line.

Table 7: The antibacterial activity of *Annona muricata* using ethanolic extract

Reading	*Bacillus subtilis*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*E.coli*
25µl	1.0cm	1.2cm	1.0cm	0.5cm
50µl	1.5cm	1.3cm	0.8cm	0.8cm
75µl	1.0cm	1.7cm	1.6cm	1.0cm
100µ	2.0cm	1.4cm	1.5cm	1.5cm
Standard	1.2cm	1.5cm	1.3cm	0.5cm

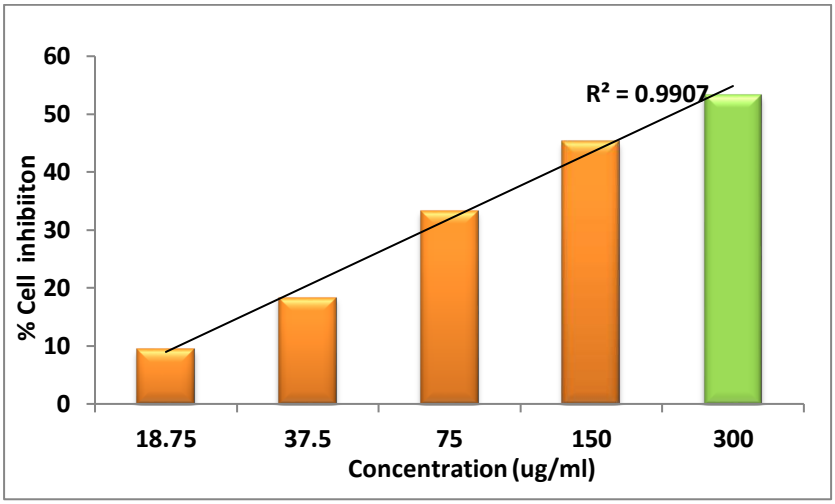

Figure 4. Anticancer property of *Anonna muricata* by MTT assay

Annonna Muricata. L extracts can inhibit proliferation and cause apoptosis of various types of cancer cells in in-vitro as well as in mice models in-vivo, and can suppress tumor metastasis [38] which shows that *Anonna Muricata* provides an option for cancer ethno medicinal therapy. Our result confirms that the leaf extract can cause apoptosis of liver cancer cells as studied by MTT assay.

CONCLUSION

The present study demonstrated that *Annona muricata* is a good source of various phytochemicals like alkaloids, flavonoids, carbohydrates, glycosides, saponins, tannins, terpenoids and proteins. Antioxidant activity was studied, which may be useful in the prevention of cancer. The antibacterial activity of *Annona muricata* extract was evaluated against various test organisms like *Staphylococcus*, *Pseudomonas, Bacillus,* and *E. coli*. According to anticancer study of *Annona muricata* shows an effective property. The anticancer molecules present in this plant were used for therapeutic purposes. These preliminary results warrant an in-depth study is needed to evaluate the usefulness of the miracle plant components for the treatment of various diseases.

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